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ISCHEMIA ACTIVATES NEUTROPHILS BUT INHIBITS THEIR LOCAL AND  
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neutrophils were treated with phorbol myristate acetate (PMA)  $10^{-7}$  M. In contrast to a 162% increase in oxidative burst prior to ischemia, neutrophils at 10 minutes of reperfusion had an enhanced response to PMA of 336% ( $p < 0.05$ ). Plasma collected from the ischemic hindlimb at 10 minutes of reperfusion when introduced into an abraded skin chamber or intratracheally induced diapedesis in non-ischemic animals. PMN accumulations in the skin chamber were  $1636 \pm 258$  PMN/mm<sup>3</sup> after 3 hours ( $n = 8$ ) compared to  $63 \pm 18$  PMN/mm<sup>3</sup> induced by sham plasma ( $n = 4$ ,  $p < 0.05$ ). Introduction of ischemic plasma intratracheally into a lobar bronchus ( $n = 4$ ) induced PMN accumulations after 3 hours, measured by bronchoalveolar lavage fluid of  $19 \pm 2 \times 10^4$  PMN/mm<sup>3</sup> compared to  $5 \pm 1 \times 10^4$  PMN/mm<sup>3</sup> with sham plasma ( $n = 4$ ,  $p < 0.05$ ). Diapedesis was completely prevented ( $0-3$  PMN/mm<sup>3</sup>,  $p < 0.05$ ) by introducing ischemic plasma into skin chambers in animals whose hindlimbs had been made ischemic ( $n = 6$ ) or into chambers located on skin regions which had been previously made ischemic ( $n = 6$ ). Similarly, following hindlimb ischemia, lavage of the lung with ischemic plasma yielded few PMN  $0-3$ /mm<sup>3</sup> ( $p < 0.05$ ). These data indicate that ischemia and reperfusion lead to generation of a circulating component in plasma that causes an oxidative burst in PMN and inhibits their diapedesis but when applied extravascularly to a naive animal promotes diapedesis.

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### ABSTRACT

Hindlimb ischemia and reperfusion results in local limb and distant lung injury. This study tests whether the mechanism of injury is by ischemia mediated polymorphonuclear leukocyte (PMN) activation and diapedesis. Anesthetized rabbits were subjected to three hours of hindlimb ischemia (n=8) or sham ischemia (n=4). PMN derived solely from the reperfused ischemic limb, assayed flow cytometrically displayed an oxidative burst of  $135 \pm 8$  femtomoles dichlorofluorescein (fmDCF)/cell compared to pre-ischemic levels of  $74 \pm 14$  fmDCF/cell ( $p < 0.05$ ). Additional aliquots of isolated neutrophils were treated with phorbol myristate acetate (PMA)  $10^{-7}$ M. In contrast to a 162% increase in oxidative burst prior to ischemia, neutrophils at 10 minutes of reperfusion had an enhanced response to PMA of 336% ( $p < 0.05$ ). Plasma collected from the ischemic hindlimb at 10 minutes of reperfusion when introduced into an abraded skin chamber or intratracheally induced diapedesis in non ischemic animals. PMN accumulations in the skin chamber were  $1636 \pm 258$  PMN/mm<sup>3</sup> after 3 hours (n=8) compared to  $63 \pm 18$  PMN/mm<sup>3</sup> induced by sham plasma (n=4,  $p < 0.05$ ). Introduction of ischemic plasma intratracheally into a lobar bronchus (n=4) induced PMN accumulations after 3 hours, measured by bronchoalveolar lavage fluid of  $19 \pm 2 \times 10^4$  PMN/mm<sup>3</sup> compared to  $5 \pm 1 \times 10^4$  PMN/mm<sup>3</sup> with sham plasma (n=4,  $p < 0.05$ ). Diapedesis was completely prevented ( $0 - 3$  PMN/mm<sup>3</sup>,  $p < 0.05$ ) by introducing ischemic plasma into skin chambers in animals whose hindlimbs had been made ischemic (n=6) or into chambers located on skin regions which had been previously made ischemic (n=6). Similarly, following hindlimb ischemia, lavage of the lung with ischemic plasma yielded few PMN  $0 - 3$ /mm<sup>3</sup> ( $p < 0.05$ ). These data indicate that ischemia and reperfusion lead to generation of a circulating component in plasma that causes an oxidative burst in PMN and inhibits their

diapedesis but when applied extravascularly to a naive animal promotes diapedesis.

## INTRODUCTION

Ischemia and reperfusion induce polymorphonuclear leukocyte (PMN) dependent tissue injury. Thus, following lower torso ischemia, there is a prominent accumulation of leukocytes in, but not outside of the pulmonary microvasculature (1). Neutrophils are not seen in the lung interstitium and cannot be recovered by bronchoalveolar lavage (BAL). Further, neutrophils are not found with light microscopy in the tissue of the reperfused lower torso. Despite the physical absence of neutrophils in the interstitium it is believed that these cells mediate increased microvascular permeability (2). We postulate that the reperfused tissue generates a factor(s) which enhances PMN activation, induces PMN adhesion in the microcirculation, but inhibits local and remote diapedesis.

To test this hypothesis, the PMN oxidative burst was assayed flow cytometrically following hindlimb ischemia. Further, plasma collected during reperfusion was introduced into abraded skin chambers or intratracheally into non-ischemic animals to measure its effects on diapedesis. The same studies were conducted in ischemic animals to evaluate possible inhibition of PMN accumulations.

## METHODS

Animal preparation. Forty-seven New Zealand white male rabbits weighing 3 to 6 kg were used. Initial anesthesia was achieved with intramuscular ketamine (35 mg/kg) and intravenous xylazine (5 mg/kg) and maintained with xylazine 2 mg/kg every 30 minutes. Saline, 0.3 ml/kg/h was infused via a carotid arterial cannula placed aseptically via a small neck incision on the day of the experiment. All animals were placed on 37° C heating pads.

**Hindlimb ischemia.** Anesthetized rabbits (n=8) underwent 3 hours of bilateral hindlimb tourniquet ischemia. Cuffs were inflated to 160 mmHg. Prior to completion of the ischemic period, the vena cava was ligated just above the iliac confluence to ensure collection of blood derived solely from the ischemic region. Activated neutrophils are known to sequester in the lung microcirculation and if sampling were conducted from the systemic circulation might be unavailable for sampling. Moreover, lung leukosequestration following reperfusion induces eicosanoid generation. This methodology excludes nonischemic-tissue derived metabolites. During the first 10 minutes of reperfusion, the venous return of both hindlimbs was collected from the vena cava, distal to its point of ligation (approximately 0.5 ml/min) and discarded. A similar volume of saline was simultaneously replaced via the carotid line. In previous studies by our laboratory, it was found that plasma thromboxane B<sub>2</sub> and leukotriene B<sub>4</sub> levels peaked 10 minutes following reperfusion of the ischemic hindlimb, whereas immediate levels were not raised. Therefore, after the 10 minute washout, 4 ml of hindlimb venous return was collected in cooled heparinized syringes containing 0.1 ml ethylene diamine tetracetic acid (0.07 mg/ml) and aspirin (0.05 mg/ml) and transferred on ice for flow cytometry. Thereafter, another 10 ml sample of blood was collected in a similar manner and centrifuged at 1500 x g for 20 minutes. Aliquots of 0.5 ml plasma were frozen at -20° C and subsequently used in an in vivo chemotactic assay. Prior studies have shown that heparin, EDTA or aspirin in doses used in plasma did not interfere with the chemotactic assay.

**Sham rabbits.** Animals (n=4) were prepared as above but were not subjected to ischemia.

**Flow Cytometry.** Intracellular generation of H<sub>2</sub>O<sub>2</sub> by blood neutrophils was quantitated using flow cytometry and dichlorofluorescein-diacetate (DCFH). DCFH is a non-fluorescent

compound which is oxidized to the highly fluorescent dichlorofluorescein (DCF) within neutrophils undergoing a respiratory burst. Leukocytes were isolated from blood using dextran sedimentation, 6% in 0.9% saline, (0.3 ml dextran solution per 3 ml blood) for 45 minutes at room temperature (Dextran T 500, Pharmacia. Piscataway NJ). In preliminary experiments, it was found that any centrifugation, vortexing or even vigorous pipetting led to increases in baseline oxidation in neutrophils, so these procedures were eliminated in the final protocol. Aliquots of leukocyte rich sediment (0.01 ml) were added to 1 ml of balanced salt solution (BSS). This solution contained NaCl (124 mM), KCl (5.8 mM), dextrose (10 mM) and hydroxyethylpiperazine ethanesulphonic acid (20 mM) and was titrated with NaOH to pH 7.4 prior to use. The BSS also contained DCFH (Molecular Probes, Eugene OR) and either buffer or phorbol myristate acetate (PMA)  $10^{-7}$ M. The concentration of DCFH was  $100\mu\text{M}$ , an amount which saturated leukocytes in samples from sham or experimental animals. After incubation for 20 minutes at  $37^{\circ}\text{C}$  the samples were placed on ice and analyzed with an Ortho Diagnostics System 2151 Cytofluorograf flow cytometer using the 488 nm excitation line of an argon laser at 125 mw output. The PMN within each sample were identified by light scattering. After electronic gating, the green fluorescence of these cells in unstimulated and PMA stimulated samples was quantitated (3,000-5,000 neutrophils per sample). In some experiments, the fluorescence values obtained by flow cytometry were calibrated with samples of pure rat neutrophils. These cells were obtained 4 hours after intraperitoneal glycogen. The cells were suspended in BSS,  $10^6$  cells/ml and labelled with DCFH with or without PMA treatment. Measurements were conducted in a fluorometer (Perkin-Elmer, Norwalk CT). Using a standard curve constructed with reagent grade DCF (Sigma Chemical Co., St. Louis MO) the amount of DCFH oxidized to DCF by neutrophil  $\text{H}_2\text{O}_2$  was quantitated, allowing conversion of the mean fluorescence channel number to femtomoles

(fm) of DCF produced per cell, a value equivalent to fm of  $H_2O_2$  produced per cell.

Skin abrasion blister chambers. Chemotactic responses were measured by a modification of the technique of Otani (3). After anesthesia, a 20 x 25 cm area of the back of the rabbits was clipped with electric shears (Wahl Clipper Company, Sterling IL). These animals (n=24) were only used to assay chemotaxis. The clipped region was coated with sodium thioglycolate (Lemon Scented Nair, Carter Products, New York, NY) for 15 min, washed with tap water, rinsed with 100 ml of 0.25% acetic acid and then re-rinsed with tap water. The animals were permitted to rest for 24 to 36 hours to allow any non-specific inflammatory response to subside before chemotactic studies were performed. Animals exhibiting skin sensitivity were excluded from the study.

On the day of the experiment, the animal was re-anesthetized and a circular area of hairfree skin was outlined with a template having a 9/32 inch diameter (Rapi-Design Template No. 40, Rapi-Design Products, Burbank CA). This area was gently abraded with an electric ink eraser (Petty Electric Ink Eraser, Pierce Corp., River Falls, WI) until uniform glistening was produced. This normally took 15-20 seconds. The debris from the abraded area was removed by pressing adhesive tape (Blender M, 3M Surgical Products, St. Paul MN) over the site. On rare occasions, when abrasion led to trace bleeding, the site was abandoned.

Plastic, "unit dose" blister chambers (Rexhaus Corporation, Westfield Industrial Park, Westfield MA) with a volume capacity of 0.5 ml were placed over the abraded areas and secured with Steridrape (3M Surgical Products, St. Paul MN). Usually 18 to 24 blister chambers could be affixed to each rabbit. Injections into the chambers were made with a 27-gauge needle. At the conclusion of the experiment, fluid was withdrawn from the chambers and neutrophils counted with a hemocytometer. Animals were allowed to rest for 14 days before they were re-used.

Plasma Aspiration. A tracheostomy was performed with a 7 mm tube. Through this tube a fine polyethylene cannula (internal diameter, 0.64 mm Delmed, Inc., Canton, MA) was introduced into the bronchus of the middle lobe of the right lung. Evans Blue dye 0.2 mg was added to the lavage solution for later confirmation of the location of the fine cannula. One ml of ischemic or normal plasma was introduced via the cannula into the bronchus of the middle lobe of the right lung and after 5 minutes the cannula was removed. Three hours later, the animals were euthanized with an overdose of ketamine. A thoracotomy was performed and the left lung bronchus clamped. Broncho-alveolar lavage (BAL) was performed via the tracheostomy tube using three applications of 5 ml of saline. The BAL recovery of about 10 ml was centrifuged at 6000 rpm for 5 minutes (GCL-1 Centrifuge, Sorvall, Newton, CT). The pellet was suspended in 1 ml saline and PMN counted after Diff-Quik staining to identify macrophages (AHS del Caribe, Inc, Aguada, Puerto Rico).

Skin Capillary Blood Flow. This was measured using laser doppler (Laserflow Model BPM, No. 3, TSI, Inc., St. Paul, MN). The doppler signal produced an output proportional to blood flow, expressed in ml/100 g/min (4).

Experimental Protocol. Anesthetized rabbits were subjected to bilateral hindlimb ischemia (n=8) or sham ischemia (n=4) for 3 hours. After 10 minutes of reperfusion the venous effluent was collected. PMN were separated and used for flow cytometry and the plasma used for blister and intratracheal treatments. The plasma was introduced into blisters atop animals: whose hindlimbs had been made ischemic (n=9); or who had been subjected to sham ischemia (n=8); or whose dorsal skin used for the blister preparation had been made ischemic with a Satinsky clamp (n=6). Plasma collected from animals who had undergone sham ischemia was used as control in all studies. Hindlimb or skin ischemia was for a period of 3 hours. Ten minutes of reperfusion

was allowed before plasma was placed in the blister pack. In lung diapedesis studies, ischemic or sham plasma was introduced into the bronchus of the right middle lobe of animals subjected to 3 hours of hindlimb ischemia and 10 minutes of reperfusion ( $n=8$ ) or sham ischemia ( $n=4$ ).

Data are expressed as mean  $\pm$  SEM in text and figures. An analysis of variance, followed by a non-paired Student's  $t$ -test was used to determine significance between groups. Significance was accepted if  $p < 0.05$ .

Animals in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education and Welfare, Publication No. 78-23 (National Institute of Health), revised, 1978).

## RESULTS

Neutrophils derived from the reperfused hindlimb displayed an oxidative burst of  $135 \pm 8$  fmDCF/cell compared to pre-ischemic levels of  $74 \pm 14$  fmDCF/cell ( $p < 0.05$ ). Additional aliquots of isolated neutrophils treated with PMA  $10^{-7}$ M showed a 162% increase in  $H_2O_2$  production prior to ischemia and an enhanced response of 336% after ischemia ( $p < 0.05$ , Fig. 1). Ischemic plasma when introduced into abraded skin chambers or intratracheally induced diapedesis in non-ischemic animals. There were  $1636 \pm 258$  PMN/mm<sup>3</sup> in the skin chambers after 3 hours compared to  $63 \pm 18$  PMN/mm<sup>3</sup> induced by sham plasma ( $p < 0.05$ , Fig. 2). Introduction of ischemic plasma into the lung of non ischemic animals yielded by BAL, accumulations of  $19 \pm 2 \times 10^4$  PMN/mm<sup>3</sup> compared to  $5 \pm 1 \times 10^4$  PMN/mm<sup>3</sup> induced by sham plasma ( $p < 0.05$ ). In animals subjected to 3 hours of hindlimb ischemia and 10 minutes of reperfusion, the

ability of ischemic plasma to induce diapedesis in skin blisters or lungs was abolished. The recovery of PMN was  $0 - 3/\text{mm}^3$  in blister fluid and BAL ( $p < 0.05$ ). Finally, skin rendered ischemic prevented diapedesis induced by ischemic plasma introduced into blisters on the previously ischemic skin ( $0-3 \text{ PMN}/\text{mm}^3$ ,  $p < 0.05$ ).

**Skin Blood Flow.** Baseline skin blood flow, measured with the laser doppler was  $5.6 \pm 0.18 \text{ ml}/100\text{g}/\text{min}$ . During hindlimb ischemia the dorsal abraded skin region showed increased blood flow of  $6.8 \pm 0.25 \text{ ml}/100\text{g}/\text{min}$  ( $p < 0.05$ ). In one hour it had returned to baseline levels of  $5.4 \pm 0.14 \text{ ml}/100\text{g}/\text{min}$ . During skin ischemia flow was reduced to  $0.04 \pm 0.01 \text{ ml}/100\text{g}/\text{min}$  while after 1 hour of reperfusion flow increased to  $6.4 \pm 0.2 \text{ ml}/100\text{g}/\text{min}$  ( $p < 0.05$ ) and then returned to the preischemic levels of  $5.7 \pm 0.3 \text{ ml}/100\text{g}/\text{min}$ .

## DISCUSSION

Ischemia and reperfusion lead to PMN dependent injury. Recent studies have emphasized the role of PMN adhesion in modulating injury following reperfusion. Thus, leukocyte adherence receptor antibodies prevented adhesion and subsequent injury (5,6). We postulate that ischemia leads to PMN activation, manifest by an oxidative burst and adherence receptor upregulation and that both phenomena mediate the reperfusion injury.

The first part of this study was designed to test the ability of reperfused tissue to increase PMN oxidative activity. Flow cytometry of PMN derived from the venous effluent of reperfused tissue demonstrated an increase in  $\text{H}_2\text{O}_2$  production as well as an exaggerated response to a second stimulus, that is PMA. The mechanism of ischemia induced PMN activation is unknown but may be via arachidonic acid derivatives. Thus, leukotriene (LT)  $\text{B}_4$  synthesized by reperfused tissue is a chemoactivator (1). By stimulating a receptor site on the PMN surface, activation and

an oxidative burst can ensue. In addition,  $\text{LTB}_4$  may stimulate leukocyte adherence receptor expression (7).

The second part of the study tested the ability of ischemic plasma to induce diapedesis. It was found that plasma derived from the ischemic hindlimb during reperfusion induced diapedesis in both abraded skin chambers as well as in the lungs of non-ischemic animals. These data suggest that reperfused tissue generates a factor(s) that increases PMN-endothelial interaction. The ability of ischemic plasma to induce diapedesis when applied extravascularly may also be leukotriene dependent. Thus, ischemic plasma contains increased levels of  $\text{LTB}_4$ , which is known to be both a powerful chemoactivator and chemoattractant.

The third part of the study tested the effect of local and remote ischemia in moderating diapedesis. Surprisingly, following ischemia of skin, ischemic plasma with a known chemotactic ability was unable to induce diapedesis when instilled into blister chambers positioned on this previously ischemic skin region. Similarly, following hindlimb ischemia, ischemic plasma introduced by lavage into the lung or into the blister packs failed to induce diapedesis. It has been reported that mixing PMN with a chemoattractant will inhibit subsequent directed migration (13). This is consistent with our observations that the intravascular administration of a chemoactivator induced by ischemia and reperfusion will inhibit a later chemotactic stimulus. These results suggest a desensitization of the neutrophil. Other interpretations are possible. Thus, chemoactivators released intravenously may stimulate endothelium to synthesize prostaglandins which could serve as inhibitors of PMN adhesion and diapedesis. This is unlikely, since neither indomethacin nor aspirin pretreatment of endothelial cells, in order to inhibit prostaglandin synthesis prevented neutrophil adhesion (9). Secondly, activated endothelial cells may generate a non cyclooxygenase dependent leukocyte adhesion inhibitor (10). Reports indicate that such an

inhibitor acts directly on the leukocyte, is blocked by actinomycin D but not by indomethacin or aspirin. However, it is unlikely that ischemic tissue synthesizes a leukocyte adhesion inhibitor, since the time scale of synthesis of this stable protein is 4 hours (10). In our study, there was complete inhibition of diapedesis over the course of the first three hours after ischemia. Finally, a low flow phenomenon following hindlimb ischemia, induced by PMN plugging of the microcirculation (11,12) of the skin or lung is an unlikely cause of the inhibition of diapedesis. Histology of ischemic reperfused tissue (1) and skin blood flow measurements reported in this study and by others (13) discount this mechanism.

We speculate that the extravascular administration into lungs or blister pack of ischemic plasma will activate endothelium to synthesize intercellular adhesion molecules (ICAM) and/or endothelial leukocyte adhesion molecules (ELAM). Thus, *in vitro* or *in vivo* preincubation of endothelium with a chemoattractant increases PMN adhesion and diapedesis, events associated with ICAM or ELAM synthesis (14-17). In contrast, *in vitro* preincubation of PMN with leukotriene B<sub>4</sub>, similar in effect to intravascular leukotriene B<sub>4</sub> administration or ischemia and reperfusion, will decrease PMN diapedesis through EC (18). This desensitization suggests leukocyte adhesion receptor downregulation, or alternatively failure of induction of endothelial ICAM or ELAM. A recent study has described the sequence of neutrophil activation following hindlimb ischemia (19). The oxidative burst and prominent additional response to PMA stimulation are noted after 5 minutes. At 30 minutes of reperfusion the neutrophils cannot be stimulated by PMA whereas at 60 minutes the PMA effect has been restored to levels equal to those noted before ischemia. These results indicate that the timing of the second stimulus is important. It is possible that the chemotactic effect, blunted by hindlimb ischemia would be restored after several hours. In a clinical setting, repeated episodes of ischemia may lead to long term inhibition

of chemotaxis and therefore vulnerability to sepsis. The observation of neutrophil migration into regions of myocardial or intestinal ischemia is not necessarily at variance with these considerations. It is likely that neutrophil desensitization is temporary (18) and with the continued presence of an extravascular chemoattractant, neutrophils will ultimately migrate.

Adhesion without diapedesis of activated neutrophils is sufficient to induce lung injury, documented by increased permeability. Studies in this laboratory have described the role of adhesion in mediating tissue injury following hindlimb ischemia. Eicosanoids, particularly leukotrienes and thromboxane  $A_2$ , as well as oxygen free radicals appear to be important since their pharmacologic antagonists prevent lower torso ischemia induced lung leukosequestration and microvascular permeability increase (20-24).

In summary, these data indicate that ischemia and reperfusion lead to the generation of a circulating agent in plasma which results in a PMN oxidative burst and inhibition of diapedesis. This is likely due to deactivation of the stimulated neutrophil since ischemic plasma, when applied extravascularly, enhances PMN migration.

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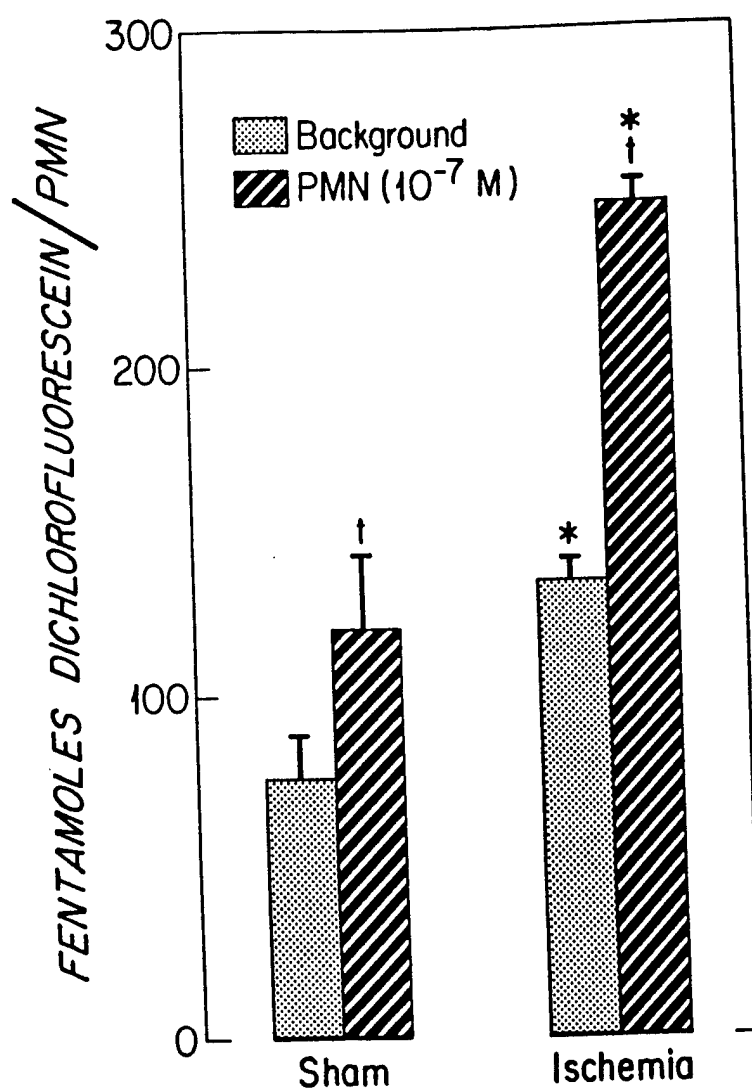
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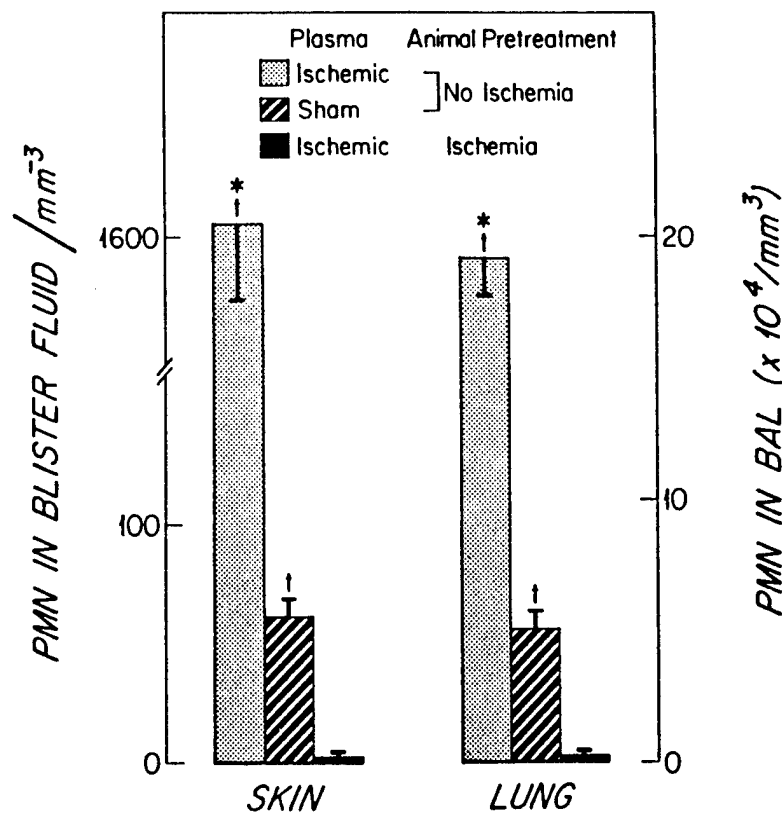
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**Figure 1.** Neutrophil  $\text{H}_2\text{O}_2$  production measured flow cytometrically was enhanced following ischemia and by treatment with phorbol myristate acetate. Asterisks and daggers refer to significance ( $p < 0.05$ ) between and within groups.



**Figure 2.** PMN counts in skin blisters and bronchoalveolar lavage fluid were increased by ischemic plasma relative to sham plasma in animals who had not been made ischemic ( $p < 0.05$ ) as indicated by asterisks. In contrast, when animals were subjected to hindlimb ischemia, diapedesis into skin blisters or lung was prevented even compared to sham plasma ( $p < 0.05$ ) as indicated by daggers.



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